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Herbal medicine Sho-saiko-to (TJ-9) prevents liver fibrosis and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet

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Background/Aim: **A herbal medicine, Sho-saiko-to (TJ-9), has recently been orally administered to patients with chronic liver disease in Japan and has been reported to inhibit the development of hepatocellular carcinoma. The aim of this study was to investigate whether TJ-9 has an inhibitory effect on the development of preneoplastic lesions and liver fibrosis in rats.**

Methods: **The effects of the TJ-9 were examined using the choline-deficient L-amino acid-defined (CDAA) diet-induced liver fibrosis model in 16-week-old male Wistar rats.**

Results: **TJ-9 (1% w/w) prevented fibrosis, as indicated by reduced hydroxyproline content in the liver and inhibition of the increase in a serum marker of fibrosis (hyaluronic acid), without reducing the increase in serum alanine aminotransferase and aspartate aminotransferase. TJ-9 also reduced the expression of type III procollagen alpha 1 mRNA in the liver, as well as the proliferation of myofibroblast-like cells (activated stellate cells, activated Ito cells). Furthermore, TJ-9 reduced the number of preneoplastic lesions, detected as enzyme-altered (glutathione S-transferase placental form-positive) lesions, in the liver.**

Conclusions: **These results indicate that the herbal medicine Sho-saiko-to (TJ-9) prevents fibrosis as well as preneoplastic lesions, not by inhibiting hepatocyte cell death but by inhibiting the activation of stellate cells, which are considered to be the main collagen-producing cells, leading to a reduction in the development of preneoplastic lesions.**

Key words: **Carcinogenesis; Fibrosis; Herbal medicine; Preneoplastic lesion; TJ-9.**

INTERFERON α and β have been studied as possible therapeutic agents for the eradication of virus in patients with chronic viral hepatitis, thus preventing liver fibrosis. However, the results of such studies have generally been unsatisfactory. Chronic viral hepatitis (especially type C) in Japan (1) and hemochromatosis lead to the development of hepatocellular carcinoma, usually in a fibrotic or cirrhotic liver (2).

A herbal medicine, Sho-saiko-to (TJ-9), has recently been orally administered to patients with chronic liver disease in Japan and has been found to inhibit the development of hepatocellular carcinoma (3). Recent *in vivo* and *in vitro* studies have reported its effects as an anti-tumor drug and as a biological response modulator (4–7). We have reported that the prevention of fibrosis may reduce the possibility of developing hepatocellular carcinoma by inhibiting the activation of stellate (Ito or fat-storing) cells, which are considered the main collagen-producing cells, in rat liver cirrhosis induced by administering a choline-deficient L-amino acid-defined (CDAA) diet (8–10).

In the present study, using this CDAA-diet model, we investigated the mechanism and effects of TJ-9 on the extent of liver fibrosis and preneoplastic lesions as assessed by enzyme-altered lesions. Our results showed that TJ-9 prevented liver fibrosis and the development of preneoplastic lesions by directly inhibiting the activation of stellate cells.

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Materials and Methods

Animals

Male Wistar rats, 6 weeks of age and weighing 140–150 g, (Nippon SLC Co., Ltd., Shizuoka, Japan), were obtained, quarantined for 1 week, and housed in a room under controlled temperature (25°C), humidity, and lighting (12 h light, 12 h dark). Access to food and tap water was *ad libitum* throughout the study period. After a 1-week acclimatization period on a basal diet (Oriental MF Diet, Oriental Yeast Company, Japan), the rats were divided into experimental groups.

Diets

The choline-deficient L-amino acid-defined (CDAA) and choline-supplemented L-amino acid-defined (CSAA) diets were obtained in powdered form (Dyets, Inc., Bethlehem, PA, USA; product numbers 518753, 518754). The detailed compositions of these diets have been described in previous reports (8–10). Briefly, the amino acid composition of the CDAA and CSAA diets consisted of only pure L-amino acids (with the exception of glycine, which is not optically active). All diets contained 50 g/kg of corn oil and 100 g/kg of Primex as a lipid source. The average caloric contents of the CDAA and CSAA diets were 4.32 and 4.27 kcal/g, respectively. The CDAA diet contained 6.5 mg/kg of choline and 1.75 g/kg of methionine. The CSAA diet was supplemented with 14.48 g/kg of choline. The total amino acid (protein) contents of the CDAA and CSAA diets were the same. TJ-9 in powdered form was mixed uniformly into the CDAA or CSAA diet at 1% (w/w) concentration.

The bioavailable choline in 1% (w/w) TJ-9 was 17.8 mg/kg of choline bitartrate, as measured by high-performance liquid chromatography (HPLC). We therefore added 17.8 mg/kg of choline bitartrate to the CDAA diet to obtain a modified CDAA (m-CDAA) diet. Diets with or without TJ-9 were stored at 4°C immediately after preparation and were consumed within 2 weeks.

Experimental protocol

The total study period was 16 weeks. The 5 experimental groups for assessing the effect of TJ-9 on hydroxyproline content comprised 30 or 10 rats each, with rats housed 5 per cage. Three groups of 30 rats each received a CDAA diet alone, an m-CDAA diet alone, or a CDAA diet containing 1% TJ-9. Two groups of 10 rats each received a CSAA diet with or without 1% TJ-9. In order to equalize the total food intake in all groups, additional food was not supplied until all food in all groups had been consumed.

Net food intake of the CDAA, m-CDAA, and CDAA+1% TJ-9 diets was measured and the mean±SD of total food intake was calculated for each cage of 5 rats over 16 weeks. For rats fed a CSAA or CSAA+1% TJ-9 diet, the mean total food intake over 16 weeks for 2 cages was measured.

At the end of the study, all rats were killed under ether anesthesia. Blood was obtained from the bifurcation of the abdominal aorta and the liver was excised. The livers were weighed, then immediately frozen for hydroxyproline measurements or fixed in 10% formalin for 24 h and embedded in paraffin for type III collagen, α-smooth muscle actin, and glutathione S-transferase placental form (GST-P) staining.

Serum markers

After 16 weeks, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and hyaluronic acid levels were determined using a previously reported assay (10).

Histology and immunohistochemical examination

Sections of 5-μm thickness of the right lobe of all rat livers were processed routinely for hematoxylin and eosin staining. Type III collagen, α-smooth muscle actin for the detection of activated stellate cells, and GST-P-positive lesions (as preneoplastic lesions) were immunohistochemically assessed by the avidin-biotin-peroxidase complex method, as previously described (8,10). Rabbit anti-rat type III collagen polyclonal antibody (Cosmo Bio, Inc., Tokyo, Japan) and anti-α-smooth muscle actin monoclonal antibody (Dako Japan, Inc., Kyoto, Japan) were employed.

For the morphometric semi-quantitative analysis of activated stellate cells in the livers of rats fed a CDAA diet, an m-CDAA diet, or a CDAA diet with TJ-9 for 16 weeks, we measured α-smooth muscle actin-positive cells in 6 ocular fields per specimen as percent area at 40×magnification using an image analysis system (Personal Image Analysis System LA-555; Pias, Ltd., Osaka, Japan). The number of α-smooth muscle actin-positive cells was expressed as a percentage of the total area of the specimen, as previously described (10). In addition, the area of GST-P-positive lesions was measured using the same system and expressed as a percentage of the total area of the specimen, as previously described (8).

Electron microscopy

Morphological changes in stellate cells were also examined by electron microscopy. Liver tissue specimens were sliced into small pieces and fixed with 2.5% glutaraldehyde and 1% $OsCl_4$. Then, the tissues were dehydrated, substituted by propylene, and embedded in epoxy resin. Thin sections were double-

stained with uranyl acetate and lead citrate and then examined by transmission electron microscopy (H-800, Hitachi, Tokyo, Japan), as previously described (10).

Hydroxyproline content

Hydroxyproline content was determined by the modified Kivirikko method, as previously reported (10). Briefly, liver specimens were weighed and 20 mg of freeze-dried sample was hydrolyzed in 6-M HCl at 110°C in an autoclave at a pressure of 1.2 kgf/cm^2 for 24 h. After centrifugation at 2000 rpm at a temperature of 4°C for 5 min, 2 ml of supernatant was mixed with 50 ml of 1% phenolphthalein and 8-N KOH to obtain a total volume of 5 ml liquid at pH 7–8. Then, 2 ml of this solution was stirred with 2 g of KCl and 1 ml of 0.5-M borate buffer (pH 8.2) for 15 min at room temperature and for another 15 min at 0°C, after which 1 ml of 0.2-M chloramine T solution was added and stirred for 60 min at 0°C. After addition of 2 ml of 3.6-M sodium thiosulfate, the solution was incubated for 30 min at 120°C and stirred with 3 ml of toluene for 20 min. Next, 0.8 ml of Ehrlich's solution was added to 2 ml of supernatant after centrifugation at 2000 rpm at 4°C and left for 30 min at room temperature. Absorbance was measured at 560 nm. The hydroxyproline content of the liver was expressed as μg/g wet weight.

Probes

The following probes were used in this study. A 700-b EcoR1/Hind III fragment of the complementary deoxynucleic acid (cDNA) of type III procollagen alpha 1 excised from the pBr322 clone (11) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (12), purchased from American Type Culture Collection (Rockville, MD, USA).

Northern blot analysis

Total RNA was isolated from the liver tissue by extraction of guanidine isothiocyanate and centrifugation in cesium chloride (13). Poly(A)$^+$ RNA was selected by oligo(dt)-cellular chromatography (14). Five micrograms of poly(A)$^+$ RNA from each sample were electrophoresed in 1% agarose gel containing 0.66 mol/l formaldehyde and ethidium bromide (0.66 μg/ml). Then, Northern blot analysis was carried out as previously described (10).

Statistical methods

Results are expressed as mean±SD, and the data obtained were evaluated by ANOVA as appropriate. The level of significance was set at 5% for each analysis.

Ethical considerations

This experiment was reviewed by the Committee for Ethics in Animal Experiments of Yamaguchi University School of Medicine and carried out under the Guidelines for Animal Experiments of Yamaguchi University School of Medicine and Law No. 105 and Notification No. 6 of the Japanese Government.

Results

Hydroxyproline content of the liver

As 1% (w/w) of TJ-9 has been reported to be effective against the development of hepatic foci induced by N-nitrosomorpholine (15), we used this concentration of TJ-9 to examine its effects on liver fibrosis and preneoplastic (GST-P-positive) lesions induced by a CDAA diet.

Rats fed a CDAA diet and rats fed an m-CDAA diet for 16 weeks (n=30 in each group) showed increased liver hydroxyproline contents of 546±134 and 563±151 μg/g wet weight, respectively, compared with 147±43 μg/g wet weight for rats fed a CSAA diet (n=10). The addition of 17.8 mg/kg of choline bitartrate, which is equivalent to the choline content in 1% TJ-9, to the CDAA diet to produce the m-CDAA diet had no effect on the hydroxyproline content of the liver. The administration of 1% TJ-9 significantly (p<0.01) reduced the hydroxyproline content of the liver (411±171 μg/g wet weight, n=30) compared with values observed in rats fed the CDAA or m-CDAA diet (Table 1). The addition of 1% TJ-9 to the CSAA diet had no effect on the hydroxyproline content of the liver (144±52 μg/g, n=10) (Table 1).

There was no difference in total food intake among the experimental groups (Table 2), and the weight gain of the rats in this experiment was almost same as that previously observed in rats fed a CSAA or CDAA diet *ad libitum* (data not shown). This indicates that caloric restriction did not influence the results obtained for the rats in any of the experimental groups.

Serum markers of liver fibrosis

Hyaluronic acid has also been reported to reflect the extent of liver fibrosis (16). In this model, rats fed a CDAA diet for 16 weeks showed an increased serum hyaluronic acid level of 76±47 ng/ml, compared with 35±8 ng/ml for rats fed a CSAA diet. Concurrent administration of 1% TJ-9 significantly reduced this increase in hyaluronic acid to 55±26 ng/ml, in parallel with the reduction in hydroxyproline content (Table 1). In addition, 1% TJ-9 did not reduce the increase in serum ALT level (Table 1) or AST level (data not shown). Thus, the inhibition of fibrosis by TJ-9 cannot be attributed to the prevention of hepatocyte cell in-

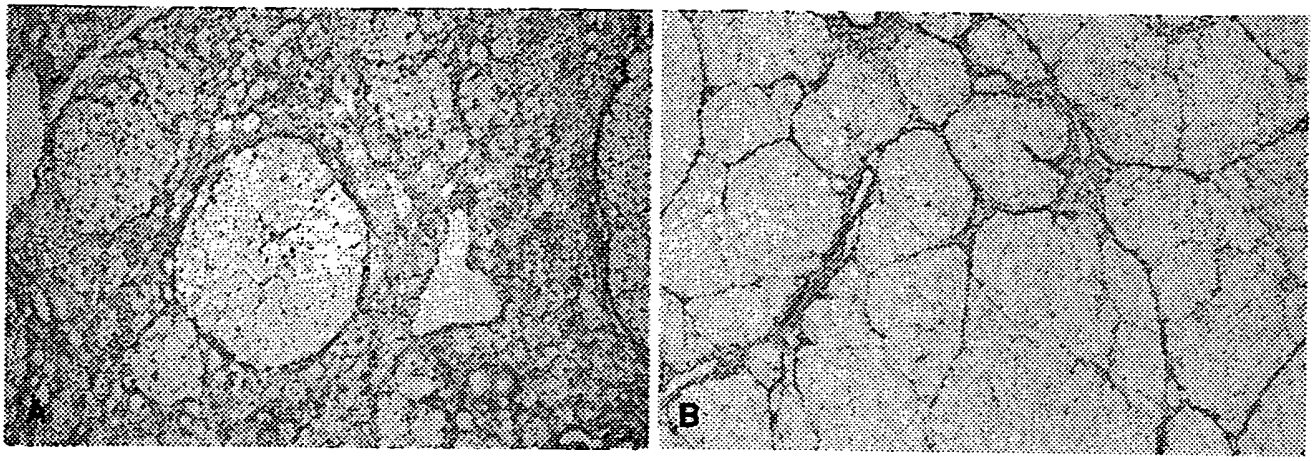

Fig. 1. Photomicrographs of liver sections stained with anti-rat type III collagen antibody from a male Wistar rat fed an m-CDAA diet for 16 weeks (A) and from a rat fed a CDAA diet with 1% TJ-9 for 16 weeks (B). Magnification 100×.

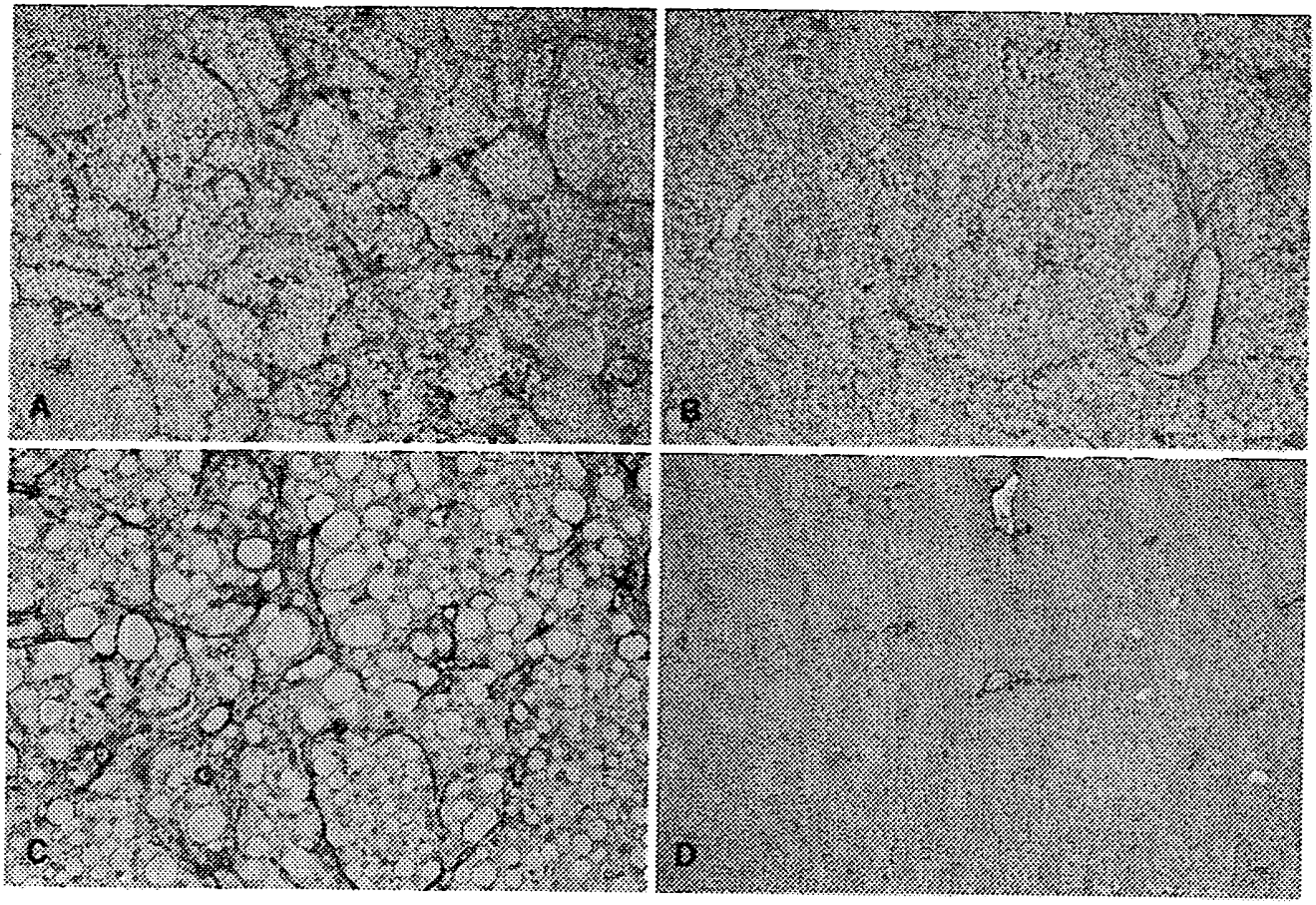

Fig. 2. Photomicrographs of liver sections stained with anti-rat α-smooth muscle actin antibody from a male Wistar rat fed an m-CDAA diet for 16 weeks (A,C), from a rat fed a CDAA diet with 1% TJ-9 for 16 weeks (B), and from a rat fed a CSAA diet for 16 weeks (D). Magnification: A, B, D 100×; C 400×.

jury resulting in fibrosuppression, and this increase in serum ALT may reflect the residual hepatocytes present in the fibrotic liver resulting from the prevention of fibrosis by TJ-9, as shown in a previous report (10). The administration of 1% TJ-9 to rats fed a CSAA diet did not affect serum ALT and AST levels (data not

TABLE 1

Effect of TJ-9 on various markers

Treatment (no. of rats)	Hydroxyproline (μg/g wet wt)	α-Smooth muscle actin-positive cells (%)	GST-P-positive lesions (%)	Hyaluronic acid (ng/ml)	ALT (U/l)
CDAA (30)	546±134	3.59±2.36	3.42±3.85	76±47	97±33
m-CDAA (30)	563±151	3.88±2.61	4.81±3.84	65±31	118±78
CDAA+1% TJ-9 (30)	411±171*‡	2.80±2.23*‡	1.58±1.75*‡	55±26§	140±52*
CSAA (10)	147±43	0.13±0.04	0	35±8	24±5
CSAA+1% TJ-9 (10)	144±52	0.12±0.04	0	33±9	27±6

Note: Values are mean±SD.
* $p<0.01$ vs. CDAA.
‡ $p<0.01$ vs. m-CDAA.
§ $p<0.05$ vs. CDAA.
Hydroxyproline was measured as described in the text. Percent area of α-smooth muscle actin-positive cells was assessed using an image analysis system as follows: the number of α-smooth muscle actin-positive cells in 6 ocular fields per specimen was assessed as percent area at 40× magnification. The percent area of GST-P-positive lesions was also assessed using an image analysis system, as described in the text.

shown). These findings indicate that 1% TJ-9 did not exhibit any hepatotoxicity.

Histological findings

The livers of rats fed an m-CDAA diet or a CDAA diet for 16 weeks showed extensive accumulation of type III collagen, representing the extracellular matrix (Fig. 1A). Co-administration of 1% TJ-9 prevented the accumulation of type III collagen (Fig. 1B), similar to the findings of reduced hydroxyproline content in the liver (Table 1). Activated stellate cells, which express α-smooth muscle actin and are therefore also called myofibroblast-like cells, showed marked proliferation in the livers of rats fed an m-CDAA diet or a CDAA diet for 16 weeks (Fig. 2A). The addition of 1% TJ-9 reduced the number of α-smooth muscle actin-positive cells in the liver (Fig. 2B). Also, semi-quantitative analysis showed that 1% TJ-9 significantly reduced the number of α-smooth muscle actin-positive cells compared with a CDAA diet or an m-CDAA diet (Table 1). The cells stained by anti-α-smooth muscle actin monoclonal antibody in the livers of rats fed a CSAA or CSAA+1% TJ-9 diet were restricted to the vessels, as shown in Fig. 2D. The values obtained in these specimens were very small and seemed not to be related to the values observed in rats fed a CDAA, m-CDAA, or CDAA+1% TJ-9 diet (Table 1). These findings suggest that the prevention of fibrogenesis by TJ-9 is related to its inhibitory effect on the activation of stellate cells.

Transmission electron microscopy also revealed that many stellate cells in the livers of rats fed a CDAA diet alone or an m-CDAA diet alone for 16 weeks shared the same characteristic morphology, i.e., few fat droplets and extended rough endoplasmic reticulum, the so-called myofibroblast-like cell (activated stellate cell) (Fig. 3A) (17,18). Administration of 1% TJ-9 tended to prevent the loss of fat droplets and reduced the extent of rough endoplasmic reticulum, with cells showing morphological characteristics similar to those of cells in the resting state (Fig. 3B), as seen in the livers of rats fed a CSAA diet.

Northern blot hybridization of mRNA in the liver

A 5.4-kb segment of α_1(III) procollagen transcript was clearly demonstrated by the Northern-blot analysis of poly $(A)^+$ RNA isolated from the livers of rats fed an m-CDAA diet alone or a CDAA diet with 1% TJ-9 (Fig. 4). The levels of α_1(III) procollagen mRNA expression were markedly increased in the livers of rats fed an m-CDAA diet alone for 16 weeks, compared with the livers of rats fed a CSAA diet, in which expression was not detected (data not shown). Densitometric analysis of α_1(III) procollagen, as shown in Fig. 4, after standardization against the expression of G3PDH, indicated that the expression of α_1(III) procollagen in rats fed a CDAA+1% TJ-9 diet was sig-

TABLE 2

Body weight and food intake

Treatment (no. of rats)	Initial body wt (g)	Final body wt (g)	Net food intake (g/rat)
CDAA (30)	152±4	318±18*	1644±11
m-CDAA (30)	152±5	311±24*	1645±14
CDAA+1% TJ-9 (30)	152±5	308±19‡	1641±12
CSAA (10)	153±3	365±19	1651
CSAA+1% TJ-9 (10)	153±4	368±18	1640

Values are mean±SD.
* $p<0.01$ vs. CSAA.
‡ *NS* vs. CDAA and m-CDAA.
Net food intake of CDAA, m-CDAA, and CDAA+1% TJ-9 diets was measured as mean±SD of total food intake for each cage of 5 rats over 16 weeks. For rats fed a CSAA or CSAA+1% TJ-9 diet, mean total food intake over 16 weeks for 2 cages was measured.

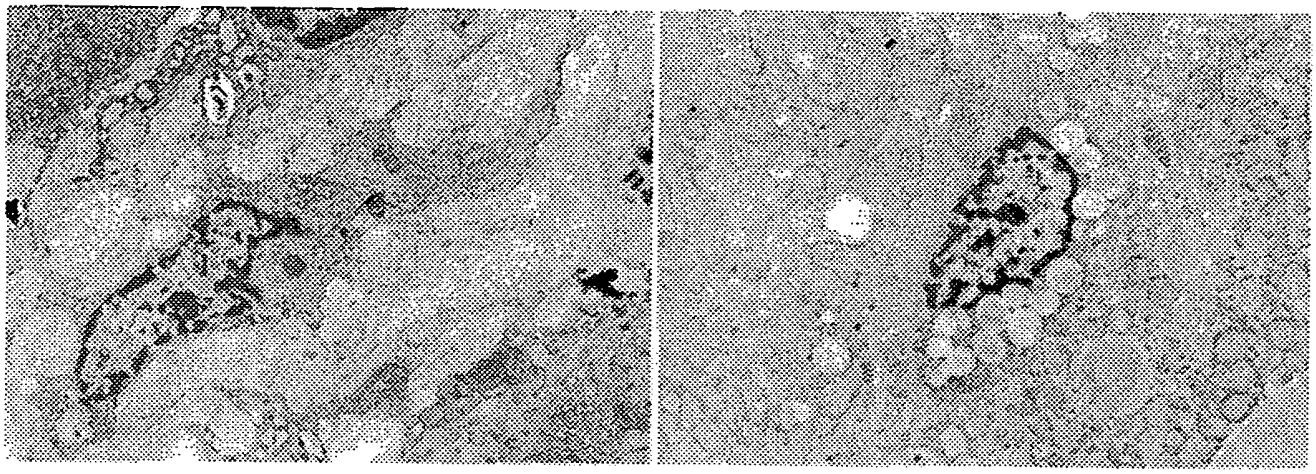

Fig. 3. Electron micrographs of representative stellate cells in the livers of rats fed an m-CDAA diet (A) or a CDAA diet with 1% TJ-9 (B) for 16 weeks. (A) Fat droplets are rarely seen, and the rough endoplasmic reticulum is markedly extended. (B) Fat droplets remain, and extension of the rough endoplasmic reticulum is not as marked as in (A).

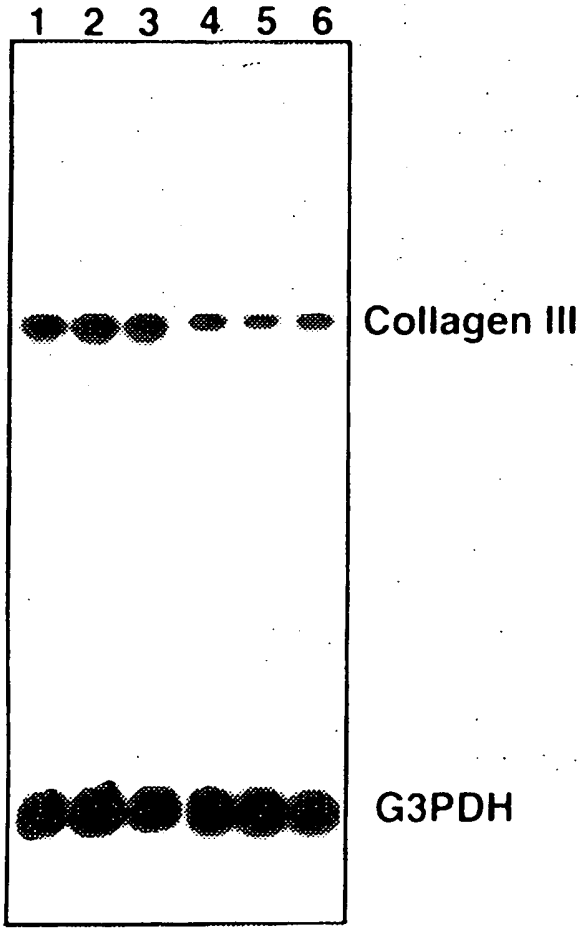

Fig. 4. Messenger RNA expression of α_1 (III) procollagen and G3PDH in the livers of rats fed an m-CDAA diet alone (lanes 1, 2, 3) or a CDAA diet with 1% TJ-9 (lanes 4, 5, 6) for 16 weeks. The figure shows a representative example of 3 independent Northern blots.

nificantly ($p<0.05$) reduced compared to that in rats fed an m-CDAA diet (0.157±0.03 vs. 0.195±0.04, $n=9$ in each group). There was no significant difference in α_1(III) mRNA expression in the livers of rats fed an m-CDAA diet or a CDAA diet (data not shown).

Thus, the prevention of fibrosis by TJ-9 can presumably be attributed to the prevention of procollagen gene expression.

Effect of TJ-9 on GST-P-positive lesions

Typical GST-P-positive nodules surrounded by fibrous septa are shown in Fig. 5. In this model at 16 weeks, GST-P-positive lesions consisted mainly of these nodules.

The results of semi-quantitative analysis of GST-P-positive lesions in the liver at the end of the study are summarized in Table 1. Administration of a CDAA diet for 16 weeks was associated with the development of a large number of GST-P-positive lesions. The concomitant administration of 1% TJ-9 significantly reduced the area of GST-P-positive lesions, in parallel with the reduction in hydroxyproline content, compared with the livers of rats fed a CDAA or an m-CDAA diet. GST-P-positive lesions in rats fed a CDAA+1% TJ-9 diet (Fig. 5B) tended to be smaller nodules, but administration of 1% TJ-9 did not significantly affect the histological findings other than the reduction in the formation of fibrous septa, e.g., liver necrosis and fatty liver as well as inflammatory cells (which are rarely seen), as previously reported (8). The relation between α-smooth muscle actin-positive cells and GST-P-positive nodules was also examined, and a significant correlation was observed, as shown in Fig. 6.

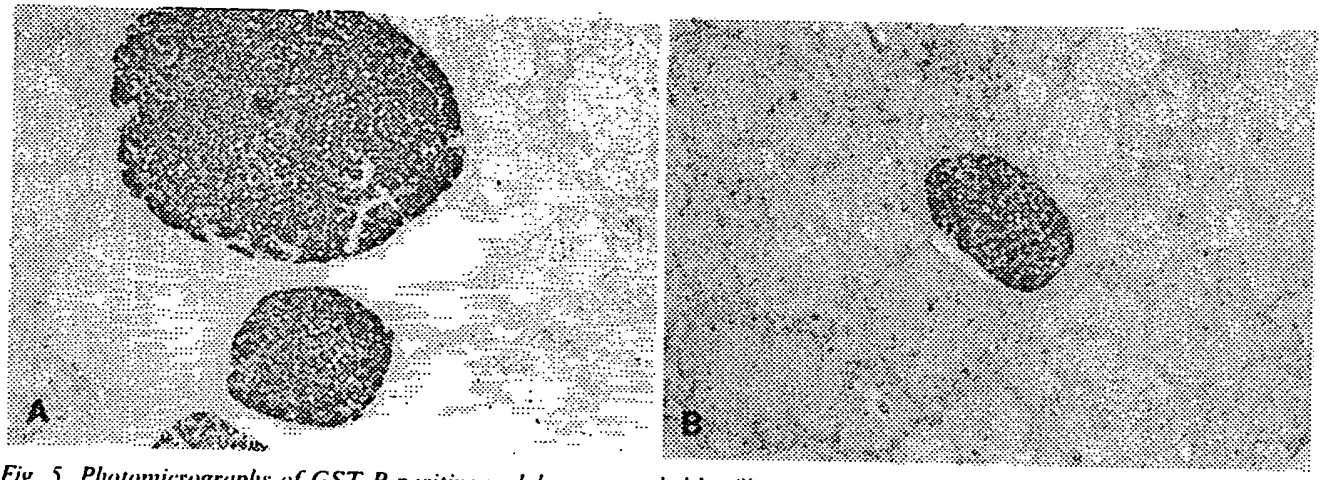

Fig. 5. Photomicrographs of GST-P-positive nodules surrounded by fibrous septa in a liver section from a male Wistar rat fed an m-CDAA diet (A) and a section from a male Wistar rat fed a CDAA diet with 1% TJ-9 (B) for 16 weeks. Magnification 100×.

Discussion

In this study, oral administration of 1% TJ-9 significantly prevented the development of GST-P-positive lesions in parallel with a reduction in the hydroxyproline content of the liver in CDAA diet-induced or m-CDAA diet-induced liver cirrhosis (Table 1). Because the choline content of 1% (w/w) TJ-9 is equivalent to 17.8 mg/kg of choline bitartrate, this amount of choline was added to the CDAA diet to produce the m-CDAA diet as an accurate experimental control.

The hydroxyproline content of the liver in rats fed a CDAA diet with 1% TJ-9 was significantly lower than that in rats fed a CDAA diet or an m-CDAA diet for 16 weeks. Thus, the fibrosuppressive effect of 1% TJ-9 cannot be attributed to the additional choline it contributed to the CDAA diet. Another possibility is the inhibition of free radical processes by TJ-9, because hepatocyte cell death induced by a CDAA diet is related to free radical processes (19). However, the increase in serum ALT and AST levels was not reduced in rats fed a CDAA diet with TJ-9 (Table 1). Thus, 1% TJ-9 had no protective effect against free radical-related hepatocyte cell injury in the CDAA-diet model. In fact, the serum ALT and AST levels in rats fed a CDAA diet with 1% TJ-9 were higher than those in rats fed a CDAA diet alone or an m-CDAA diet. This may be due to the residual hepatocytes present in the less fibrotic liver, and the inhibition of fibrosis by TJ-9 cannot be attributed to a direct action in preventing liver cell injury, as was previously reported for this experimental fibrosis model (10). The administration of 1% TJ-9 to rats fed a CSAA diet did not increase the serum ALT and AST levels (Table 1), indicating that this agent is safe and has no hepatotoxic effect.

In this CDAA-diet model of liver cirrhosis, 1% TJ-9 prevented the accumulation of hydroxyproline, which reflects the amount of collagen in the liver (Table 1). This prevention of hydroxyproline accumulation was in agreement with the reduced level of serum hyaluronic acid, as a marker of fibrosis (Table 1).

Histological findings also supported the prevention of liver fibrosis by TJ-9 in the CDAA-diet model (Fig. 1A and B). Administration of 1% TJ-9 did not notably change the histological findings, e.g., fatty changes, other than the reduced formation of fibrous septa. The expression of α-smooth muscle actin has been reported to be a marker of stellate cell activation (20,21). The administration of 1% TJ-9 significantly prevented the activation of stellate cells (Fig. 2A and B), as assessed by anti-α-smooth muscle actin antibody, which stains

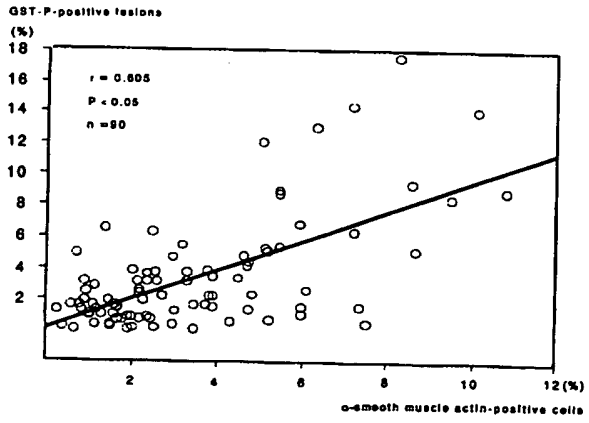

Fig. 6. The relationship between GST-P-positive lesions and α-smooth muscle actin-positive cells in the liver of rats fed a CDAA, m-CDAA, and CDAA+1% TJ-9 diet (n=90).

these cells only when they are activated, and electron microscopic findings (Fig. 3A and B). Semi-quantitative analysis showed that the addition of 1% of TJ-9 significantly reduced the number of α-smooth muscle actin-positive cells (activated stellate cells) compared with that seen in the livers of rats fed a CDAA or m-CDAA diet (Table 1). Moreover, as shown in Fig. 4, 1% TJ-9 significantly reduced type III procollagen mRNA expression in the livers of rats fed a CDAA diet for 16 weeks compared with rats fed an m-CDAA diet, which is an accurate experimental control. Thus, the point of fibrosuppression by TJ-9 appears to be at the level of procollagen mRNA.

The stellate cell is now considered to be the main collagen-producing cell under non-physiological conditions (22–25). Our results suggest that TJ-9 prevented the accumulation of collagen proteins, presumably by inhibiting the activation of stellate cells, as indicated by the expression of α-smooth muscle actin-positive cells (Fig. 2) as well as electron microscopic findings (Fig. 3), leading to reduced expression of procollagen mRNA in proportion to the reduced production of collagens, including type III (Fig. 4).

With regard to the serum hyaluronic acid level, there was no significant difference between rats fed an m-CDAA diet and those fed a CDAA diet with 1% TJ-9. However, there was at least a tendency towards a decrease, and 1% TJ-9 in a CDAA diet did reduce the hydroxyproline content and mRNA expression in the liver, which are more important for the evaluation of fibrosis, compared with an m-CDAA diet. Thus, these data indicate that TJ-9 has a direct inhibitory effect on stellate cell activation. TJ-9 has also been observed to reduce the expression of α_1 (III) and α_2 (I) procollagen mRNAs as well as the proliferation of isolated stellate cells. At least one of the components of TJ-9, baicalein, has a direct inhibitory effect on stellate cell activation, and other components are currently under investigation (Kayano et al., unpublished data, July 1997).

Finally, the prevention of preneoplastic lesions (GST-P-positive lesions) by TJ-9 may be attributable to the prevention of liver fibrosis by the inhibition of stellate cell activation, as previously reported (Table 1) (8). After 16 weeks on a CDAA diet alone, GST-P-positive lesions mainly consisted of nodules surrounded by fibrous septa, resulting in the formation of pseudolobuli (Fig. 5). The liver hydroxyproline content reflects the amount of collagen fibers making up the fibrous septa (26). Thus, the inhibition of GST-P-positive lesions by TJ-9 can be presumed to be attributable to the prevention of pseudolobule formation by fibrous septa. In addition, the inhibition of carcinogenesis by TJ-9 could be attributed to, for example, the prevention of DNA hypomethylation, which is thought to be one possible mechanism of carcinogenesis (27,28). Because fibrosis (pseudolobule formation) may interfere with methyl delivery through blood flow, the reduction of fibrosis (pseudolobule formation) could reduce DNA damage and GST-P-positive lesions. The significant relation observed between α-smooth muscle actin-positive cells and GST-P-positive nodules (Fig. 6) also supports the hypothesis that fibrosuppression by inhibition of stellate cells prevents the development of GST-P-positive nodules. However, TJ-9 has a number of effects on the immune system (29), and these effects on preneoplastic lesions could be the result of its activity as a biological response modifier. Although immunological mechanisms could not be completely excluded by architecture and the absence of inflammatory cells, the presence of inflammatory cells, though rare, with or without TJ-9 in the CDAA diet model suggests that the inhibitory effect of TJ-9 on preneoplastic lesions is not due to its effect on the immune system. Moreover, the mechanism of cell death induced by a choline-deficient diet is now thought to be the result of oxidative stress, as described above (19).

Although another possibility is that the inhibitory effect of TJ-9 is attributable to its anti-tumor effect, as previously reported (3–6,30), the effect of TJ-9 on preneoplastic lesions remains unknown, and long-term experiments to determine whether TJ-9 actually prevents the development of neoplasms in the liver are required. However, our data suggest that the chemopreventive effect of TJ-9 on human hepatocellular carcinoma (3) can be attributed to the prevention of fibrosis (8), and TJ-9 is one of the few drugs currently available which is effective in interferon-unresponsive patients with chronic hepatitis.

In summary, the herbal medicine Sho-saiko-to (TJ-9) prevented liver fibrosis in choline-deficient diet-induced liver cirrhosis in rats, presumably by inhibiting stellate cell activation, resulting in the reduced development of preneoplastic lesions.

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References

1. Liver Cancer Study Group of Japan. Survey and follow-up study of primary liver cancer in Japan – report 10. Kanzou 1993; 15: 805–13.
2. Fargion S, Mandelli C, Piperno A, Ceasana R, Fracanzani AL, Fraquelli M, et al. Survival and prognostic factors in 212

Italian patients with genetic hemochromatosis. Hepatology 1992; 15: 655–9.

3. Oka H, Yamamoto S, Kuroki T, Harihara S, Marumo T, Kim SR, et al. Prospective study of chemoprevention of hepatocellular carcinoma with Sho-saiko-to (TJ-9). Cancer 1995; 76: 743–9.
4. Matsuzaki Y, Kurokawa F, Terai S, Matsumura Y, Kobayashi N, Okita K. Cell death induced by baicalein in human hepatocellular carcinoma cell lines. Jpn J Cancer Res 1996; 87: 170–7.
5. Okita K, Kurokawa F, Yamasaki T. Possible prevention of hepatocarcinogenesis with Sho-saiko-to. Gastroenterology (Tokyo) 1990; 12: 152–6.
6. Yano H, Mizoguchi A, Fukuda K, Haramaki M, Ogasawara S, Momosaki S, et al. The herbal medicine Sho-saiko-to inhibits proliferation of cancer cell lines by inducing apoptosis and arrest at the G0/G1 phase. Cancer Res 1994; 54: 448–54.
7. Hasegawa I, Mizoguchi Y, Tsutsui H, Ichikawa Y, Kawada N, Morisawa S, et al. Effects of Sho-saiko-to on interleukin 6 synthesis from mouse spleen cells. J Med Pharm Soc 1990; 7: 24–7.
8. Sakaida I, Kubota M, Kayano K, Takenaka K, Mori K, Okita K. Prevention of fibrosis reduces enzyme-altered lesions in the rat liver. Carcinogenesis 1994; 15: 2201–6.
9. Sakaida I, Okita K. New prolyl 4-hydroxylase inhibitor reduces procollagen gene expression and enzyme-altered lesions in rat liver cirrhosis. J Gastroenterol Hepatol 1995; 10(suppl.): 63–4.
10. Sakaida I, Matsumura Y, Kubota M, Kayano K, Takenaka K, Okita K. The prolyl 4-hydroxylase inhibitor HOE 077 prevents activation of Ito cells, reducing procollagen gene expression in rat liver fibrosis induced by choline-deficient L-amino acid-defined diet. Hepatology 1996; 23: 755–63.
11. Chu M-L, Weil D, Wei W, Bernard M, Sippola M, Ramirez F. Isolation of cDNA and genomic clones encoding human pro-α_1(III) collagen. J Biol Chem 1985; 260: 4357–63.
12. Tso JY, Sun X, Kao T, Reece T, Wu R. Isolation and characterization of rat and human glyceraldehyde-3 phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. Nucleic Acids Res 1985; 13: 2485–2502.
13. Schweizer J, Goerttler K. Synthesis *in vivo* of keratin polypeptides directed by mRNA isolation from newborn and adult mouse epidermis. Eur J Biochem 1980; 112: 243–9.
14. Aviv H, Leder P. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA 1972; 69: 1408–12.
15. Tatsuta M, Iishi H, Baba M, Nakaizumi A, Uehara H. Inhibition by Xiao-chai-hu-tang (TJ-9) of development of hepatic foci induced by N-nitrosomorpholine in Sprague-Dawley rats. Jpn J Can Res 1991; 82: 987–92.
16. Ueno T, Inuzuka S, Torimura T, Tamaki S, Koh H, Kin M, et al. Serum hyaluronate reflects hepatic sinusoidal capillarization. Gastroenterology 1993; 105: 475–81.
17. Horn T, Junge J, Christoffersen P. Early alcoholic liver injury: activation of lipocytes in acinar zone 3 and correlation to degree of collagen formation in the Disse space. J Hepatol 1986; 3: 333–40.
18. Chen W, Gendrault J-L, Steffan A-M. Isolation, culture and main characteristics of mouse fat-storing cells: interaction with viruses. Hepatology 1989; 9: 352–62.
19. Ghoshal AK, Farber E. Biology of disease: choline deficiency, lipotrope deficiency and the development of liver disease including liver cancer: a new perspective. Lab Invest 1993; 68: 255–60.
20. Rocky DC, Boyles JK, Gabbiani G, Friedman SL. Rat hepatic lipocytes express smooth muscle actin upon activation *in vivo* and in culture. J Submicrosc Cytol Pathol 1992; 24: 193–203.
21. Rocky DC, Maher JJ, Jarnagin WR, Gabbiani G, Friedman SL. Inhibition of rat hepatic lipocyte activation in culture by interferon-γ. Hepatology 1992; 16: 776–84.
22. Friedman SL. The cellular basis of hepatic fibrosis. N Engl J Med 1993; 328: 1828–35.
23. Milani S, Herbst H, Schuppan D, Surrenti C, Riecken EO, Stein H. Cellular localization of type I, III, and IV procollagen gene transcripts in normal and fibrotic human liver. Am J Pathol 1990; 137: 59–70.
24. Pietrangelo A, Gualdi R, Casalgrandi G, Geerts A, Bleser PD, Montosi G, et al. Enhanced hepatic collagen type I mRNA expression into fat-storing cells in a rodent model of hemochromatosis. Hepatology 1994; 19: 714–21.
25. Bedossa P, Houglum K, Trautwein C, Holstege A, Chojkier M. Stimulation of collagen α_1(I) gene expression is associated with lipid peroxidation in hepatocellular injury: a link to tissue fibrosis? Hepatology 1994; 19: 1262–71.
26. Nakano N. Morphogenesis of septa in hepatic fibrosis induced by choline deficiency in rats. Acta Pathol Jpn 1986; 36: 1643–52.
27. Wilson MJ, Shivapurkar N, Poirier LA. Hypomethylation of hepatic nuclear DNA in rats fed with a carcinogenic methyl-deficient diet. Biochem J 1984; 218: 987–90.
28. Wainfan E, Dizik M, Stender M, Christman JK. Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl-deficient diets. Cancer Res 1989; 49: 4094–7.
29. Oda T, Oka H. Kampo preparation as biological response modifiers in the treatment of chronic viral hepatitis. In: Hosaya E, Yamamura Y, editors. Recent Advances in the Pharmacology of Kampo (Japanese Herbal) Medicine. Tokyo: Excerpta Medica; 1988. p. 369–77.
30. Okita K, Li Q, Murakami A, Takahashi M. Anti-growth effects with component of Sho-saiko-to (TJ-9) on cultured human hepatoma cells. Eur J Cancer Prev 1993; 2: 169–76.